

FIELD OF INVENTION

BACKGROUND TO THE INVENTION

Human Parainfluenza virus types 1,2,3 and Respiratory syncytial virus types A and B are the major viral pathogens responsible for causing severe respiratory tract infections in infants and young children. It is estimated that, in the United States alone, approximately 1.6 million infants under one year of age will have a clinically significant RSV infection each year and an additional 1.4 million infants will be infected with PIV-3. Approximately 4000 infants less than one year of age in the United States die each year from complications arising from severe respiratory tract disease caused by infection with RSV and PIV-3. The WHO

and NIALD vaccine advisory committees ranked RSV number two behind HIV for vaccine development while the preparation of an efficacious PIV-3 vaccine is ranked in the top ten vaccines considered a priority for vaccine development.

Safe and effective vaccines for protecting infants against these viral infections are not available and are urgently required. Clinical trials have shown that formaldehyde-inactivated and live-attenuated viral vaccines failed to adequately protect vaccinees against these infections. In fact, infants who received the formalin-inactivated RSV vaccine developed more serious lower respiratory tract disease during subsequent natural RSV infection than did the control group. [Am. J. Epidemiology 89, 1969, p.405-421; J. Inf. Dis. 145, 1982, p.311-319]. Furthermore, RSV glycoproteins purified by immunoaffinity chromatography using elution at acid pH induced immunopotential in cotton rats. [Vaccine, 10(7), 1992, p.475-484]. The development of efficacious PIV-3 and RSV vaccines which do not cause exacerbated pulmonary disease in vaccinees following injection with wild-type virus would have significant therapeutic implications. It is anticipated that the development of a single recombinant immunogen capable of simultaneously protecting infants against diseases caused by infection with both Parainfluenza and Respiratory syncytial viruses could significantly reduce the morbidity and mortality caused by these viral infections.

It has been reported that a protective response against PIV-3 and RSV is contingent on the induction of neutralizing antibodies against the major viral surface glycoproteins. For PIV, these protective immunogens are the HN protein which has a molecular weight of 72 kDa and possesses both hemagglutination and neuraminidase activities and the fusion (F) protein, which has a molecular weight of 65 kDa and which is responsible for

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both fusion of the virus to the host cell membrane and cell-to-cell spread of the virus. For RSV, the two major immunogenic proteins are the 80 to 90 kDa G glycoprotein and the 70 kDa fusion (F) protein. The G and F proteins are thought to be functionally analogous to the PIV HN and F proteins, respectively. The PIV and RSV F glycoproteins are synthesized as inactive precursors (FO) which are proteolytically cleaved into N-terminal F2 and C-terminal F1 fragments which remain linked by disulphide bonds.

Recombinant surface glycoproteins from PIV and RSV have been individually expressed in insect cells using the baculovirus system [Ray et al., (1989), *Virus Research*, 12: 169-180; Coelingh et al., (1987), *Virology*, 160: 465-472; Wathen et al., (1989), *J. of Inf. Dis.* 159: 253-263] as well as in mammalian cells infected with recombinant poxviruses [Spriggs, et al., (1987), *J. Virol.* 61: 3416-3423; Stott et al., (1987), *J. Virol.* 61: 3855-3861]. Recombinant antigens produced in these systems were found to protect immunized cotton rats against live virus challenge. More recently, hybrid RSV F-G [Wathan et al., (1989), *J. Gen Virol.* 70: 2625-2635; Wathen, published International Patent application WO 89/05823] and PIV-3 F-HN [Wathen, published International Patent Application WO 89/10405], recombinant antigens have been engineered and produced in mammalian and insect cells. The RSV F-G hybrid antigen was shown to be protective in cotton rats [Wathan et al., (1989), *J. Gen. Virol.* 70: 2637-2644] although it elicited a poor anti-G antibody response [Connors et al., (1992), *Vaccine* 10: 475-484]. The protective ability of the PIV-3 F-HN protein was not reported in the published patent application. These antigens were engineered with the aim to protect against only the homologous virus, that is either RSV or PIV-3. However, it would be advantageous and economical to engineer and produce a single

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recombinant immunogen containing at least one protective antigen from each virus in order simultaneously to protect infants and young children against both PIV and RSV infections. The chimeric proteins provided herein for such purpose also may be administered to pregnant women or women of child bearing age to stimulate maternal antibodies to both PIV and RSV. In addition, the vaccine also may be administered to other susceptible individuals, such as the elderly.

10 SUMMARY OF INVENTION

In its broadest aspect, the present invention provides a multimeric hybrid gene, comprising a gene sequence coding for an ^{immunogenic} ~~antigenic~~ region of a protein from a first pathogen linked to a gene sequence coding for an ^{immunogenic} ~~antigenic~~ region of a protein from a second pathogen and to a chimeric protein encoded by such multimeric hybrid gene. Such chimeric protein comprises an ^{immunogenic} ~~antigenic~~ region of a protein from a first pathogen linked to an ^{immunogenic} ~~antigenic~~ region of a protein from a second pathogen.

a 20 The first and second pathogens ~~generally~~ are selected from bacterial and viral pathogens and, in one embodiment, may both be viral pathogens. Preferably, the first and second pathogens are selected from those causing different respiratory tract diseases, which may be upper and lower respiratory tract diseases. In a preferred embodiment, the first pathogen is parainfluenza virus and the second pathogen is respiratory syncytial virus. The PIV protein particularly is selected from PIV-3 F and HN proteins and the RSV protein particularly is selected from RSV G and F proteins. Another aspect of the invention provides cells containing the multimeric hybrid gene for expression of a chimeric protein encoded by the gene. Such cells may be bacterial cells, mammalian cells, insect cells, yeast cells or fungal cells. Further, the present invention provides a live vector for antigen delivery containing the multimeric

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child-bearing age, and other susceptible persons, such as the elderly.

The chimeric protein provided herein also may be used as a diagnostic reagent for detecting infection by a plurality of different pathogens in a host, using a suitable assaying procedure.

It will be appreciated that, while the description of the present invention which follows focuses mainly on a chimeric molecule which is effective for immunization against diseases caused by infection by PIV and RSV, nevertheless the invention provided herein broadly extends to any chimeric protein which is effected for immunization against diseases caused by a plurality of pathogens, comprising an antigen from each of the pathogens linked in a single molecule, as well as to genes coding for such chimeric molecules.

In this application, by the term "multimeric hybrid genes" we mean genes encoding antigenic regions of proteins from different pathogens and by the term "chimeric proteins" we mean immunogens containing antigenic regions from proteins from different pathogens.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the nucleotide (SEQ ID No: 1) and amino acid (SEQ ID No: 2) sequence of a PCR-amplified PIV-3 F gene and F protein, respectively;

Figure 2 shows the restriction map of the PIV-3 F gene;

Figure 3 shows the nucleotide (SEQ ID No: 3) and amino acid (SEQ ID No: 4) sequences of the PIV-3 HN gene and HN protein, respectively;

Figure 4 shows the restriction map of the PIV-3 HN gene;

Figure 5 shows the nucleotide (SEQ ID No: 5) and amino acid (SEQ ID No: 6) sequences of the RSV F gene and RSV F protein, respectively;

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Figure 7 shows the nucleotide (SEQ ID No: 7) and amino acid (SEQ ID No: 8) sequences of the RSV G gene and RSV G protein, respectively;

Figure 9 shows the steps involved in the construction of an expression vector containing a
10 chimeric $F_{PRV-3} - F_{RSV}$ gene;

15 Figure 11 shows the steps involved in the construction of an expression vector containing a chimeric F_{PIV-3} - F_{RSV} gene containing a truncated PIV-3 F gene devoid of 5'-untranslated region linked to a truncated RSV F1 gene;

Figure 13 shows immunoblots of cell lysates from Sf9 cells infected with recombinant baculoviruses;

30 Figure 15 shows the steps involved in construction
of a chimeric F_{RSV} - HN_{PIV-3} gene;

Figure 17 illustrates mutagenesis of a PIV-3 F gene;
35 and

Figure 18 shows the steps involved in the construction of a chimeric F_{PIV-3} - G_{RSV} gene.

GENERAL DESCRIPTION OF INVENTION

5 In the present invention, a chimeric molecule protective against two different major childhood diseases is provided. The present invention specifically relates to the formulation of various recombinant Parainfluenza virus (PIV)/Respiratory syncytial virus (RSV) immunogens
10 to produce safe and efficacious vaccines capable of protecting infants and young children, as well as other susceptible individuals, against diseases caused by infection with both PIV and RSV. However, as described above, the present invention extends to the construction
15 of multimeric hybrid genes containing genes coding for protective antigens from many pathogens. Such vaccines may be administered in any desired manner, such as a readily-injectable vaccine, intranasally or orally.

In the present invention, the inventors have
20 specifically engineered several model PIV/RSV chimeric genes containing relevant sequences from selected genes coding for PIV-3 and RSV surface glycoproteins linked in tandem. All genes in the chimeric constructs described herein were obtained from recent clinical isolates of
25 PIV-3 and RSV. The chimeric gene constructs may include gene sequences from either PIV-3 F or HN genes linked in tandem to either RSV F or G genes in all possible relative orientations and combinations.

The chimeric gene constructs provided herein may
30 consist of either the entire gene sequences or gene segments coding for immunogenic and protective epitopes thereof. The natural nucleotide sequence of these genes may be modified by mutation while retaining antigenicity and such modifications may include the removal of
35 putative pre-transcriptional terminators to optimize their expression in eukaryotic cells. The genes were

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designed to code for hybrid PIV-RSV surface glycoproteins linked in tandem in a single construct to produce gene products which elicit protective antibodies against both parainfluenza and respiratory syncytial viruses. Such multimeric hybrid genes consist of a gene sequence coding for a human PIV-3 F or HN protein or an immunogenic epitope-containing fragment thereof linked to a gene sequence coding for a human RSV G or F protein or an immunogenic epitope-containing fragment thereof. Specific gene constructs which may be employed include $F_{PIV-3} - F_{RSV}$, $F_{RSV} - HN_{PIV-3}$ and $F_{PIV-3} - G_{RSV}$ hybrid genes.

In addition, the present invention also extends to the construction of other multimeric genes, such as trimeric genes containing PIV and RSV genes or gene segments, linked in all possible relative orientations. For example:

$F_{PIV} - HN_{PIV} - F$ or G_{RSV}

$F_{PIV} - F_{RSV} - G_{RSV}$

$HN_{PIV} - F_{RSV} - G_{RSV}$

The multimeric genes provided herein also may comprise at least one gene encoding at least one immunogenic and/or immunostimulating molecule.

The multimeric hybrid genes provided herein may be sub-cloned into appropriate vectors for expression in cellular expression systems. Such cellular expression systems may include bacterial, mammalian, insect and fungal, such as yeast, cells.

The chimeric proteins provided herein also may be presented to the immune system by the use of a live vector, including live viral vectors, such as recombinant poxviruses, adenoviruses, retroviruses, Semliki Forest viruses, and live bacterial vectors, such as Salmonella and mycobacteria (e.g. BCG).

Chimeric proteins, such as a PIV/RSV chimera, present in either the supernatants or cell lysates of

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To evaluate the immunogenicity and protective ability of the chimeric proteins, suitable experimental animals are immunized with either varying doses of the purified chimeric proteins, such as the PIV/RSV chimera, and/or live recombinant vectors as described above. Such chimeric proteins may be presented to the immune system by either the use of physiologically-acceptable vehicles, such as aluminum phosphate, or by the use of delivery systems, such as ISCOMS and liposomes. The chimeras also may be formulated to be capable of eliciting a mucosal response, for example, by conjugation or association with immunotargeting vehicles, such as the cholera toxin B subunit, or by incorporation into microparticles. The vaccines may further comprise means for delivering the multimeric protein specifically to cells of the immune system, such as toxin molecules or antibodies. To further enhance the immunoprotective ability of the chimeric proteins, they may be supplemented with other immunogenic and/or immunostimulating molecules. The chimeric PIV/RSV proteins specifically described herein may be formulated with an adjuvant, such as aluminum phosphate, to produce readily-injectable vaccines for protection against the diseases caused by both PIV-3 and RSV. The chimeric proteins also may be administered intranasally or orally. The chimeric proteins may be used in test kits for diagnosis of infection by PIV-3 and RSV.

30 The invention is not limited to the preparation of chimeric PIV-3 and RSV proteins, but is applicable to the production of chimeric immunogens composed of either the entire sequences or regions of the immunogenic proteins from at least two pathogens sequentially linked in a
35 single molecule. Chimeric antigens also may be synthesized to contain the immunodominant epitopes of

several proteins from different pathogens. These chimeric antigens may be useful as vaccines or as diagnostic reagents.

SEQUENCE IDENTIFICATION

Several nucleotide and amino acid sequences are referred to in the disclosure of this application. The following table identifies the sequences and the location of the sequence:

<u>SEQ</u> <u>ID No.</u>	<u>Identification</u>	<u>Location</u>
1	Nucleotide sequence for PCR-amplified PIV-3 F gene	Fig. 1, Example 1
2	Amino acid sequence for PCR-amplified PIV-F protein	Fig. 1, Example 1
3	Nucleotide sequence for PIV-3 HN gene	Fig. 3, Example 1
4	Amino acid sequence for PIV-3 HN protein	Fig. 3, Example 1
5	Nucleotide sequence for RSV F gene	Fig. 5, Example 1
6	Amino acid sequence for RSV F protein	Fig. 5, Example 1
7	Nucleotide sequence for RSV G gene	Fig. 7, Example 1
8	Amino acid sequence for RSV G protein	Fig. 7, Example 1
9	BsrI - BamHI oligo- nucleotide cassette	Fig. 9, Example 2
10	BspHI - BamHI oligo- nucleotide cassette	Fig. 9, Example 2
11	EcoRI - Ppu MI oligo- nucleotide cassette	Fig. 9, Example 2
12	BrsI - BamHI oligo- nucleotide cassette	Fig. 10, Example 3

13	EcoRI -Bsr BI oligo-nucleotide cassette	Fig. 10, Example 3
5 14	EcoRV - EcoRI oligo-nucleotide cassette	Fig. 11, Example 5
15	EcoRV - BamHI oligo-nucleotide cassette	Fig. 14, Example 8
10 16	BspHI - BspHI oligo-nucleotide cassette	Fig. 15, Example 9
15 17	Nucleotide sequence for PIV-3 F gene	Example 15
18	Mutagenic oligo-nucleotide #2721	Fig. 17, Example 15
20 19	Nucleotide sequence for part of oligo-nucleotide #2721	Example 15
25 20	Oligonucleotide probe	Example 15

DEPOSIT INFORMATION

Certain plasmid DNAs described and referred to herein have been deposited with the American Type Culture Collection (ATCC) located at Rockville, Maryland, USA, pursuant to the Budapest Treaty and prior to the filing of this application. The deposited purified plasmids will become available to the public upon grant of this U.S. patent application or upon publication of its corresponding European patent application, whichever first occurs. The invention described and claimed herein is not to be limited in scope by the plasmid DNAs of the constructs deposited, since the deposited embodiment is intended only as an illustration of the invention. The following purified plasmids were deposited at the ATCC with the noted accession numbers on December 17, 1992:

<u>Plasmid</u>	<u>Example No.</u>	<u>Accession No.</u>
pAC DR7	5	75387
pD2RF-HN	9	75388
pD2F-G	16	75389

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Any equivalent plasmids that can be used to produce equivalent antigens as described in this application are within the scope of the invention.

EXAMPLES

5 The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of
10 the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

15 Methods for cloning and sequencing the PIV-3 and RSV genes as well as the procedures for sub-cloning the genes into appropriate vectors and expressing the gene constructs in mammalian and insect cells are not explicitly described in this disclosure but are well
20 within the scope of those skilled in the art.

Example 1:

This Example outlines the strategy used to clone and sequence the PIV-3 F, HN and RSV F, G genes (from a type A isolate). These genes were used in the construction of
25 the $F_{PIV-3} - F_{RSV}$, $F_{RSV} - HN_{PIV-3}$, and $F_{PIV-3} - G_{RSV}$ chimeric genes detailed in Examples 2 to 4, 9 and 15, respectively.

Two PIV-3 F gene clones initially were obtained by PCR amplification of cDNA derived from viral RNA extracted from a recent clinical isolate of PIV-3. Two
30 other PIV-3 F gene clones as well as the PIV-3 HN, RSV F and RSV G genes were cloned from a cDNA library prepared from mRNA isolated from MRC-5 cells infected with clinical isolates of either PIV-3 or RSV (type A isolate). The PIV-3 F (both PCR amplified and non-PCR
35 amplified), PIV-3 HN, RSV F and RSV G gene clones were sequenced by the dideoxynucleotide chain termination

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procedure. Sequencing of both strands of the genes was performed by a combination of manual and automated sequencing.

The nucleotide (SEQ ID No: 1) and amino acid (SEQ ID No: 2) sequences of the PCR amplified PIV-3 F gene and F protein, respectively, are presented in Figure 1 and the restriction map of the gene is shown in Figure 2. Sequence analysis of the 1844 nucleotides of two PCR amplified PIV-3 F gene clones confirmed that the clones were identical. Comparison of the coding sequence of the PCR-amplified PIV-3 F gene clone with that of the published PIV-3 F gene sequence revealed a 2.6% divergence in the coding sequence between the two genes resulting in fourteen amino acid substitutions.

The nucleotide sequence of the non-PCR amplified PIV-3 F gene clone differed from the PCR amplified gene clone in the following manner: the non-PCR amplified clone had ten additional nucleotides (AGGACAAAAG) at the 5' untranslated region of the gene and differed at four positions, 8 (T in PCR-amplified gene to C in non-PCR amplified gene), 512 (C in PCR-amplified gene to T in non-PCR amplified gene), 518 (G in PCR-amplified gene to A in non-PCR amplified gene) and 1376 (A in PCR-amplified gene to G in non-PCR amplified gene). These changes resulted in three changes in the amino acid sequence of the F protein encoded by the non-PCR amplified PIV-3 F gene. Serine (position 110), glycine (position 112), and aspartic acid (position 398) in the primary amino acid sequence of the F protein encoded by the PCR amplified PIV-3 F gene was changed to phenylalanine (position 110), glutamic acid (position 112) and glycine (position 398), respectively, in the primary amino acid sequence of the F protein encoded by the PCR amplified clone.

Figure 3 shows the nucleotide (SEQ ID No: 3) and amino acid (SEQ ID No: 4) sequences of the PIV-3 HN gene and protein, respectively and the restriction map of the

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gene is presented in Figure 4. Analysis of the 1833 nucleotide sequence from two HN clones confirmed that the sequences were identical. A 4.4% divergence in the coding sequence of the PIV-3 HN gene was noted when the sequence was compared to the published PIV-3 HN coding sequence. This divergence resulted in seventeen amino acid substitutions in the amino acid sequence of the protein encoded by the PIV-3 HN gene.

The nucleotide (SEQ ID No: 5) and amino acid (SEQ ID No: 6) sequences of the RSV F gene and RSV F protein, respectively, are shown in Figure 5 and the restriction map of the gene is shown in Figure 6. Analysis of the ¹⁸⁸⁶~~1887~~ nucleotide sequence from two RSV F clones verified complete sequence homology between the two clones. Comparison of this nucleotide sequence with that reported for the RSV F gene revealed approximately 1.8% divergence in the coding sequence resulting in eleven amino acid substitutions.

The nucleotide (SEQ ID No: 7) and amino acid (SEQ ID No: 8) sequences of the RSV G gene and RSV G protein, respectively, are presented in Figure 7 while the restriction map of the gene is outlined in Figure 8. Comparison of the 920 nucleotide sequence of the G gene clone with the published G sequence (type A isolate) revealed a 4.2% divergence in the nucleotide sequence and a 6.7% divergence in the amino acid sequence of the gene product. This divergence resulted in twenty amino acid substitutions.

The full-length PIV-3 F (non-PCR amplified) , PIV-3 HN, RSV F and RSV G genes were cloned into λ gt11 and subcloned into the multiple cloning site of a Bluescript M13-SK vector, either by blunt end ligation or using appropriate linkers. The PCR-amplified PIV-3 F gene was directly cloned into the Bluescript vector. The cloning vectors containing the PIV-3 F-PCR amplified, PIV-3 F non-PCR amplified, PIV-3 HN, RSV F and RSV G genes were

named pPI3F, pPI3Fc, pPIVHN, pRSVF and pRSVG, respectively.

Example 2:

This Example illustrates the construction of a
5 Bluescript-based expression vector (pMCR20) containing
the chimeric F_{PIV-3} - F_{RSV} gene. This chimeric gene
construct contains the 5' untranslated region of the PIV-
3 F gene but lacks the hydrophobic anchor and cytoplasmic
tail coding regions of both the PIV-3 and RSV F genes.
10 The steps involved in the construction of this plasmid
are summarized in Figure 9.

To prepare the PIV-3 portion of the chimeric gene
(Figure 9, step 1), the full length PIV-3 gene lacking
the transmembrane region and cytoplasmic tail coding
15 regions was retrieved from plasmid pPI3F by cutting the
polylinker with BamHI, blunt-ending the linearized
plasmid with Klenow polymerase and cutting the gene with
BsrI. A BsrI-BamHI oligonucleotide cassette (SEQ ID No:
9) containing a PpuMI site and three successive
20 translational stop codons were ligated to the truncated
1.6 Kb [BamHI]-BsrI PIV-3 F gene fragment and cloned into
the EcoRV-BamHI sites of a Bluescript M13-SK expression
vector containing the human methallothionin promoter and
the poly A and IVS sequences of the SV40 genome
25 (designated pMCR20), to generate plasmid pME1.

To engineer the RSV F gene component of the chimeric
construct (Figure 9, step 2), the RSV F gene lacking the
transmembrane region and cytoplasmic tail coding regions
was retrieved from plasmid pRSVF by cutting the
30 polylinker with EcoRI and the gene with BspHI. A
synthetic BspHI-BamHI oligonucleotide cassette (SEQ ID
No: 10) containing three successive translational stop
codons was ligated to the 1.6 Kb truncated RSV F gene and
cloned into the EcoRI-BamHI sites of the Bluescript based
35 expression vector, pMCR20 to produce plasmid pES13A.
Plasmid pES13A then was cut with EcoRI and PpuMI to

remove the leader and F2 coding sequences from the truncated RSV F gene. The leader sequence was reconstructed using an EcoRI-PpuMI oligocassette (SEQ ID No: 11) and ligated to the RSV F1 gene segment to generate plasmid pES23A.

To prepare the chimeric F_{PIV-3} - F_{RSV} gene (Figure 9, step 3) containing the 5' untranslated region of the PIV-3 F gene linked to the truncated RSV F1 gene fragment, plasmid pME1 (containing the 1.6 Kb truncated PIV-3 F gene) first was cut with PpuMI and BamHI. The PpuMI-BamHI restricted pME1 vector was dephosphorylated with intestinal alkaline phosphatase. The 1.1 Kb RSV F1 gene fragment was retrieved from plasmid pES23A by cutting the plasmid with PpuMI and BamHI. The 1.1 Kb PpuMI-BamHI RSV F1 gene fragment was cloned into the PpuMI-BamHI sites of the dephosphorylated pME1 vector to generate plasmid pES29A. This chimeric gene construct contains the 5' untranslated region of the PIV-3 F gene but lacks the nucleotide sequences coding for the hydrophobic anchor domains and cytoplasmic tails of both the PIV-3 and RSV F proteins.

Example 3:

This Example illustrates the construction of a Bluescript-based expression vector containing the PIV-3 F gene lacking both the 5' untranslated and transmembrane anchor and cytoplasmic tail coding regions. The steps involved in constructing this plasmid are outlined in Figure 10.

Plasmid pPI3F containing the full length PIV-3 F gene was cut with BamHI, blunt ended with Klenow polymerase and then cut with BsrI to remove the transmembrane and cytoplasmic tail coding regions. The Bluescript-based expression vector, pMCR20, was cut with SmaI and BamHI. A synthetic BsrI-BamHI oligonucleotide cassette (SEQ ID No: 12) containing a translational stop codon was ligated with the 1.6 Kb blunt ended-BsrI PIV-3

F gene fragment to the SmaI-BamHI restricted pMCR20 vector to produce plasmid pMpFB. The PIV-3 F gene of this construct lacked the DNA fragment coding for the transmembrane and cytoplasmic anchor domains but contained the 5' untranslated region. To engineer a plasmid containing the PIV-3 F gene devoid of both the 5' untranslated region and the DNA fragment coding for the hydrophobic anchor domain, plasmid pMpFB was cut with EcoRI and BstBI. An EcoRI-BstBI oligocassette (SEQ ID No: 13) containing the sequences to reconstruct the signal peptide and coding sequences removed by the EcoRI-BstBI cut was ligated to the EcoRI-BstBI restricted pMpFB vector to produce plasmid pMpFA.

Example 4:

This Example illustrates the construction of the chimeric F_{PIV-3} - F_{RSV} gene composed of the truncated PIV-3 F gene devoid of the 5' untranslated region linked to the truncated RSV F1 gene. The steps involved in constructing this plasmid are summarized in Figure 11.

To prepare this chimeric gene construct, plasmid pES29A (Example 2) was cut with BstBI and BamHI to release the 2.5 Kb BstBI-BamHI PIV-3 F-RSV F1 chimeric gene fragment. This BstBI-BamHI fragment was isolated from a low melting point agarose gel and cloned into the BstBI-BamHI sites of the dephosphorylated vector pMpFA to produce plasmid pES60A. This construct contained the PIV-3 F gene lacking both the 5' untranslated region and the hydrophobic anchor and cytoplasmic tail coding sequences linked to the F1 coding region of the truncated RSV F gene. This chimeric gene was subsequently subcloned into the baculovirus transfer vector (see Example 5).

Example 5:

This Example illustrates the construction of the modified pAC 610 baculovirus transfer vector containing the native polyhedrin promoter and the chimeric F_{PIV-3} - F_{RSV}

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gene consisting of the PIV-3 F gene lacking both the 5' untranslated sequence and the nucleotide sequence coding for the hydrophobic anchor domain and cytoplasmic tail linked to the truncated RSV F1 gene. Construction of this plasmid is illustrated in Figure 12.

The pAC 610 baculovirus expression vector was modified to contain the native polyhedrin promoter in the following manner. Vector pAC 610 was cut with EcoRV and BamHI. The 9.4 Kb baculovirus transfer vector lacking the EcoRV-BamHI DNA sequence was isolated from a low melting point agarose gel and treated with intestinal alkaline phosphatase. In a 3-way ligation, an EcoRV-EcoRI oligonucleotide cassette (SEQ ID No: 14) containing the nucleotides required to restore the native polyhedrin promoter was ligated with the 1.6 Kb EcoRI-BamHI truncated RSV F gene fragment isolated from construct pES13A (Example 2, step 2) and the EcoRV-BamHI restricted pAC 610 phosphatased vector to generate plasmid pES47A. To prepare the pAC 610 based expression vector containing the chimeric F_{PIV-3} - F_{RSV} gene, plasmid pES47A was first cut with EcoRI and BamHI to remove the 1.6 Kb truncated RSV F gene insert. The 2.8 Kb F_{PIV-3} - F_{RSV} chimeric gene was retrieved by cutting plasmid pES60A (Example 4) with EcoRI and BamHI. The 2.8 Kb EcoRI-BamHI chimeric gene was ligated to the EcoRI-BamHI restricted pES47A vector to generate plasmid pAC DR7 (ATCC 75387).

Example 6

This Example outlines the preparation of plaque-purified recombinant baculoviruses containing the chimeric F_{PIV-3} - F_{RSV} gene.

a Spodoptera frugiperda (Sf9) cells were co-transfected with 1.0 μ g wild-type AcMNPV DNA and 2.5 μ g of F_{PIV-3} - F_{RSV} plasmid DNA (plasmid pAC DR7 - Example 5). Putative recombinant baculoviruses (purified once by serial dilution) containing the F_{PIV-3} - F_{RSV} chimeric gene were identified by dot-blot hybridization. Lysates of

insect cells infected with the putative recombinant baculoviruses were probed with the ^{32}P -labelled $\text{F}_{\text{PIV-3}}\text{-F}_{\text{RSV}}$ chimeric gene insert. Recombinant baculoviruses were plaque-purified twice before being used for expression studies. All procedures were carried out according to the protocols outlined by M.D. Summers and G.E. Smith in "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures", Texas Agricultural Experiment Station, Bulletin 1555, 1987.

10 Example 7:

 This Example illustrates the presence of the chimeric $\text{F}_{\text{PIV-3}}\text{-F}_{\text{RSV}}$ protein in supernatants and cell lysates of infected Sf9 cells.

 Insect cells were infected with the plaque-purified recombinant baculoviruses prepared as described in Example 6 at a m.o.i. of 8. Concentrated supernatants from cells infected with the recombinant viruses were positive in a PIV-3 F specific ELISA. In addition, when lysates from ^{35}S -methioninelabelled infected cells were subjected to SDS-polyacrylamide gel electrophoresis and gels were analyzed by autoradiography, a strong band with apparent molecular weight of approximately 90 kDa was present in lysates of cells infected with the recombinant viruses but was absent in the lysates from wild-type infected cells. The presence of the chimeric $\text{F}_{\text{PIV-3}}\text{-F}_{\text{RSV}}$ protein in the lysates of cells infected with the recombinant baculoviruses was confirmed further by Western blot analysis using monospecific anti-PIV-3 F and anti-RSV F antisera and/or monoclonal antibodies (Mabs). Lysates from cells infected with the recombinant baculoviruses reacted with both anti-PIV-3 and anti-RSV antisera in immunoblots. As shown in the immunoblot of Figure 13, lysates from cells infected with either the RSV F or $\text{F}_{\text{PIV-3}}\text{-F}_{\text{RSV}}$ recombinant baculoviruses reacted positively with the anti-F RSV Mab. As expected, lysates from cells infected with wild type virus did not react

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with this Mab. In addition, only lysates from cells infected with the chimeric $F_{PIV-3} - F_{RSV}$ recombinant viruses reacted with the anti-PIV-3 F_1 antiserum.

Example 8

5 This Example illustrates modification of the baculovirus transfer vector pVL1392 (obtained from Invitrogen), wherein the polyhedrin ATG start codon was converted to ATT and the sequence CCG was present downstream of the polyhedrin gene at positions +4,5,6.
10 Insertion of a structural gene several base pairs downstream from the ATT codon is known to enhance translation. The steps involved in constructing this modified baculovirus transfer vector are outlined in Figure 14.

15 The baculovirus expression vector pVL1392 was cut with EcoRV and BamHI. The 9.5 kb restricted pVL1392 vector was ligated to an EcoRV-BamHI oligonucleotide cassette (SEQ ID No: 15) to produce the pD2 vector.

Example 9:

20 This Example illustrates the construction of the pD2 baculovirus expression vector containing the chimeric $F_{RSV} - HN_{PIV-3}$ gene consisting of the truncated RSV F and PIV-3 HN genes linked in tandem. The steps involved in constructing this plasmid are summarized in Figure 15.

25 To engineer the $F_{RSV} - HN_{PIV-3}$ gene, the RSV F gene lacking the nucleotide sequence coding for the transmembrane domain and cytoplasmic tail of the RSV F glycoprotein was retrieved from plasmid pRSVF (Example 1) by cutting the polylinker with EcoRI and the gene with
30 BspHI. The PIV-3 HN gene devoid of the DNA fragment coding for the hydrophobic anchor domain was retrieved from plasmid pPIVHN (Example 1) by cutting the gene with BspHI and the polylinker with BamHI. The 1.6 Kb EcoRI-BspHI RSV F gene fragment and the 1.7 Kb BspHI-BamHI PIV-
35 3 HN gene fragment were isolated from low melting point agarose gels. For cloning purposes, the two BspHI sites

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in the Bluescript based mammalian cell expression vector, pMCR20, were mutated. Mutations were introduced in the BspHI sites of the pMCR20 by cutting the expression vector with BspHI, treating both the BspHI restricted
 5 vector and the 1.1 Kb fragment released by the BspHI cut with Klenow polymerase and ligating the blunt-ended 1.1 Kb fragment to the blunt-ended Bluescript-based expression vector to generate plasmid pM'. Since
 10 insertion of the 1.1 Kb blunt-end fragment in the mammalian cell expression vector in the improper orientation would alter the Amp^r gene of the Bluescript-based expression vector, only colonies of HB101 cells transformed with the pM' plasmid DNA with the 1.1 Kb blunt-ended fragment in the proper orientation could
 15 survive in the presence of ampicillin. Plasmid DNA was purified from ampicillin-resistant colonies of HB101 cells transformed with plasmid pM' by equilibrium centrifugation in cesium chloride-ethidium bromide gradients. The 1.6 Kb EcoRI-BspHI RSV F and 1.7 Kb
 20 BspHI-BamHI PIV-3 HN gene fragments were directly cloned into the EcoRI-BamHI sites of vector pM' in a 3-way ligation to generate plasmid pM' RF-HN.

To restore specific coding sequences of the RSV F and PIV-3 HN genes removed by the BspHI cut, a BspHI-BspHI oligonucleotide cassette (SEQ ID No: 16) containing
 25 the pertinent RSV F and PIV-3 HN gene sequences was ligated via the BspHI site to the BspHI-restricted plasmid pM' RF-HN to produce plasmid pM RF-HN. Clones containing the BspHI-BspHI oligonucleotide cassette in
 30 the proper orientation were identified by sequence analysis of the oligonucleotide linker and its flanking regions.

To clone the chimeric F_{RSV}-HN_{PIV-3} gene into the baculovirus expression vector pD2 (Example 8), the F_{RSV}-
 35 HN_{PIV-3} truncated gene first was retrieved from plasmid pM RF-HN by cutting the plasmid with EcoRI. The 3.3 Kb F_{RSV}-

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HN_{PIV-3} gene then was cloned into the EcoRI site of the baculovirus transfer vector plasmid pD2 to generate plasmid pD2 RF-HN (ATCC 75388). Proper orientation of the 3.3 Kb EcoRI F_{RSV}-HN_{PIV-3} chimeric gene insert in
 5 plasmid pD2 RF-HN was confirmed by sequence analysis.

Example 10:

This Example outlines the preparation of plaque-purified recombinant baculoviruses containing the chimeric F_{RSV}-HN_{PIV-3} gene.

10 *Spodoptera frugiperda* (Sf9) cells were co-transfected with 1 µg wild-type AcNPV DNA and 2 µg of F_{RSV}-HN_{PIV-3} plasmid DNA (plasmid pD2 RF-HN-Example 9). Putative recombinant baculoviruses (purified once by serial dilution) containing the F_{RSV}-HN_{PIV-3} chimeric gene
 15 were identified by dot-blot hybridization. Lysates of insect cells infected with the putative recombinant baculoviruses were probed with the ³²P-labelled RSV F or PTV-3 HN gene oligonucleotide probes. Recombinant baculoviruses were plaque-purified three times before
 20 being used for expression studies. All procedures were carried out according to the protocols outlined by Summers and Smith (Example 6).

Example 11:

This Example illustrates the presence of the
 25 chimeric F_{RSV}-HN_{PIV-3} protein in supernatants of infected Sf9 and High 5 cells.

Insect cells (Sf9 and High 5), maintained in serum free medium EX401, were infected with the plaque purified recombinant baculoviruses of Example 10 at a m.o.i. of 5
 30 to 10 pfu/cell. Supernatants from cells infected with the recombinant baculoviruses tested positive for expressed protein in both the RSV-F and PIV-3 HN specific ELISAS. In addition, supernatants from infected cells reacted positively with both an anti-F RSV monoclonal
 35 antibody and anti-HN peptide antisera on immunoblots. A distinct band of approximately 105 kDa was present in the

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5 Example 12:

High 5 cells, maintained in serum free medium, were infected with the plaque purified recombinant baculoviruses of Example 10 at a m.o.i of 5 pfu/cell. The supernatant from virus infected cells was harvested 2 days post-infection. The soluble F_{RSV}-HN_{PIV-3} chimeric protein was purified from the supernatants of infected cells by immunoaffinity chromatography using an anti-HN PIV-3 monoclonal antibody. The anti-HN monoclonal antibody was coupled to CNBr-activated Sepharose 4B by conventional techniques. The immunoaffinity column was washed with 10 bed volumes of washing buffer (10mM Tris-HCl pH 7.5, 150 mM NaCl, 0.02% v/v ~~Triton X-100~~ ^{TRITON X-100}) prior to use. After sample loading, the column was washed with 10 bed volumes of washing buffer followed by 3 bed volumes of high salt buffer (10mM Tris-HCl pH 7.5, 500mM NaCl, 0.02% v/v Triton-X 100). The chimeric F_{RSV}-HN_{PIV-3} protein was eluted from the immunoaffinity column with 100 mM glycine, pH 2.5, in the presence of 0.02% ~~Triton X-100~~ ^{TRITON X-100}. Eluted protein was neutralized immediately with 1M Tris-HCl, pH 10.7.

Polyacrylamide gel electrophoretic analysis (Fig. 16, panel A) of the immunoaffinity-purified F_{RSV}-HN_{PIV-3} protein revealed the presence of one major protein band with an apparent molecular weight of 105 kDa. The purified protein reacted with both an anti-RSV F monoclonal antibody and anti-HN peptide antisera on immunoblots (Fig. 16, panel B, lanes 1 and 2, respectively).

Example 13:

This Example illustrates the immunogenicity of the $F_{RSV-HN_{PIV-3}}$ protein in guinea pigs.

Groups of four guinea pigs were injected intramuscularly with either 1.0 or 10.0 μ g of the chimeric $F_{RSV-HN_{PIV-3}}$ protein purified as described in Example 12 and adjuvanted with aluminum phosphate. Groups of control animals were immunized with either placebo, or live PIV-3 or RSV (administered intranasally). Guinea pigs were bled 2 and 4 weeks after the primary injection and boosted at 4 weeks with an equivalent dose of the antigen formulation. Serum samples also were taken 2 and 4 weeks after the booster dose. To assess the ability of the chimeric protein to elicit PIV-3 and RSV-specific antibody responses, sera samples were analyzed for the presence of PIV-3 specific hemagglutination inhibiting and neutralizing antibodies as well as RSV neutralizing antibodies. As summarized in Table 1 below (the Tables appear at the end of the disclosure), the sera of animals immunized with two 10 μ g doses of the chimeric protein had titres of PIV-3 specific hemagglutination inhibition (HAI) and PIV-3/RSV neutralizing antibodies at the 6 and 8 week time points which were equivalent to the levels obtained following intranasal inoculation with either live PIV-3 or RSV. In addition, animals immunized with only two 1 μ g doses of the chimeric protein elicited strong PIV-3 and RSV specific neutralizing antibodies. These results confirmed the immunogenicity of both the RSV and PIV-3 components of the chimeric protein and provided confirmatory evidence that a single recombinant immunogen can elicit neutralizing antibodies against both RSV and PIV-3.

Example 14:

This Example illustrates the immunogenicity and protective ability of the $F_{\text{RSV-HN}_{\text{PIV-3}}}$ protein in cotton rats.

Groups of eight cotton rats were injected intramuscularly with either 1.0 or 10.0 ug of the chimeric $F_{\text{RSV-HN}_{\text{PIV-3}}}$ protein (prepared as described in Example 12) adjuvanted with aluminum phosphate. Groups of control animals were immunized with either placebo (PBS + aluminum phosphate) or live PIV-3 or RSV (administered intranasally). Cotton rats were bled 4 weeks after the primary injection and boosted at 4 weeks with an equivalent dose of the antigen formulation. Serum samples were also taken 1 week after the booster dose. As shown in Table 2 below, data from the 4-week bleed demonstrated that both a 1 and 10 μg dose of the chimeric protein was capable of inducing a strong primary response. Reciprocal mean \log_2 PIV-3 specific HAI and PIV-3/RSV neutralizing titers were equivalent to the titres obtained with live PIV-3 and RSV. Thus, a single inoculation of the chimeric protein was sufficient to elicit neutralizing antibodies against both PIV-3 and RSV. Strong neutralizing PIV-3 and RSV titres also were observed following the booster dose (5 week bleed). These results provide additional evidence that both the RSV and PIV-3 components of the chimeric protein are highly immunogenic.

To assess the ability of the chimeric immunogen to simultaneously protect animals against both RSV and PIV-3, four cotton rats from each group were challenged intranasally with 100 TCID₅₀ units of either PIV-3 or RSV. Animals were killed 4 days after virus challenge. Virus titers were determined in lung ^{lavages} ~~homogenates~~. As shown in Table 3 below, animals immunized with either 1 or 10 μg of the chimeric $F_{\text{RSV-HN}_{\text{PIV-3}}}$ protein were completely protected against challenge with either PIV-3 or RSV. These results provide evidence that the chimeric protein

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is not only highly immunogenic but can also simultaneously protect cotton rats against disease caused by both PIV-3 and RSV infection.

Example 15:

5 This Example illustrates the construction of a Bluescript M13-SK vector containing the chimeric $F_{PIV-3}-G_{RSV}$ gene. This chimeric gene construct contains the 5' untranslated region of a mutated PIV-3 F gene but lacks the nucleotide sequence coding for the hydrophobic anchor and cytoplasmic tail domains of both a mutated PIV-3 F
10 and the native RSV G genes. The steps involved in constructing this plasmid are outlined in Figures 17 and 18.

 The first step (Fig. 17) involved in preparing the PIV-3 F component of the chimeric $F_{PIV-3}-G_{RSV}$ gene construct
15 was to eliminate the putative pre-termination sites within the 18 nucleotide long sequence 5' CAAGAAAAAGGAATAAAA 3' (SEQ ID No: 17) located between positions 857 and 874 of the non PCR-amplified PIV-3 F
20 gene and positions 847 and 864 of the PCR-amplified PIV-3 F gene (see Figure 1). To this end, the PIV-F cDNA of the non-PCR amplified PIV-3 F gene was cut at the BsaAI and EcoRI sites. The BsaAI-EcoRI PIV F gene fragment was
25 cloned into the EcoRI site of a Bluescript M13-SK vector using an EcoRI-BsaAI linker. The 857-874 target region of the PIV-3 F gene (non-PCR amplified) then was mutated by oligonucleotide-mediated mutagenesis using the method of Morinaga et al. [1984, Biotechnology 2: 636-639].
30 Plasmid pPI3Fc (Example 1) was cut with ScaI in the Amp^r gene and dephosphorylated with alkaline phosphatase (plasmid #1). A second sample of plasmid pPI3Fc was cut with BstEII and NsiI to produce a 3.9 Kb restricted plasmid, lacking the 0.9 Kb BstEII-NsiI fragment of the PIV-3 F gene (plasmid #2). A mutagenic 78-mer synthetic
35 oligonucleotide (#2721 shown in Fig. 17-SEQ ID No: 18)) containing the sequence 5' CAGGAGAAGGGTATCAAG 3' (SEQ ID

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No: 19) was synthesized to specifically mutate the 857-874 DNA segment without changing the F protein sequence. This oligonucleotide was added to plasmid DNAs #1 and #2, denatured at 100°C for 3 min. and renatured by gradual cooling. The mixture then was incubated in the presence of DNA polymerase, dNTPs and T4 ligase and transformed into HB101 cells. Bacteria containing the 1.8 Kb mutated PIV-3 F gene were isolated on YT agar plates containing 100 µg/ml ampicillin. Hybridization with the oligonucleotide probe 5' AGGAGAAGGGTATCAAG 3' (SEQ ID No: 20) was used to confirm the presence of the mutated PIV-3 F gene. The mutated gene sequence was confirmed by DNA sequencing. The plasmid containing the mutated PIV-3 gene was designated pPI3Fm.

The second step (Fig. 18) in the engineering of the chimeric gene construct involved constructing a Bluescript based vector to contain the truncated PIV-3 Fm gene lacking the nucleotide sequence coding for the transmembrane anchor domain and cytoplasmic tail of the PIV-3 F protein linked in tandem with the RSV G gene lacking both the 5' leader sequence and the nucleotide sequence coding for the transmembrane anchor domain and cytoplasmic tail of the G glycoprotein.

To prepare this chimeric gene, the orientation of the mutated PIV-F gene in plasmid pPI3Fm first was reversed by EcoRI digestion and religation to generate plasmid pPI3Fmr. To prepare the PIV-3 F gene component of the chimeric gene, plasmid pPI3Fmr was cut with NotI and BsrI to release the 1.7 Kb truncated PIV-3 F gene. To prepare the RSV G component, the 0.95 Kb RSV-G gene lacking both the 5' leader sequence and the DNA segment encoding the G protein anchor domain and cytoplasmic tail was released from plasmid pRSVG (Example 1) by cutting the polylinker with EcoRI and the gene with BamHI. The 0.95 Kb EcoRI-BamHI RSV G gene fragment was subcloned into the EcoRI-BamHI sites of a restricted Bluescript

vector, pM13-SK, to produce plasmid pRSVGt. The 0.95 Kb EcoRI-BamHI G gene fragment and the 1.5 Kb NotI-BsrI truncated PIV-3 F gene were linked via a BsrI-BamHI oligonucleotide cassette (SEQ ID No: 9) restoring the F and G gene coding sequences and cloned into the pRSVGt vector restricted with BamHI and NotI in a 3-way ligation. The plasmid thus generated was designated pFG.

Example 16:

This Example outlines the construction of the pD2 baculovirus transfer vector (described in Example 8) containing the chimeric $F_{PIV-3}-G_{RSV}$ gene consisting of a mutated PIV-3 F gene lacking the hydrophobic anchor and cytoplasmic coding regions linked to the RSV G gene lacking both the 5' leader sequence and the nucleotide sequences encoding the transmembrane anchor domain and cytoplasmic tail of the G protein.

To prepare this construct, plasmid pFG (Example 15) was cut with EcoRI to release the 2.6 Kb $F_{PIV-3}-G_{RSV}$ chimeric gene. The 2.6 Kb EcoRI restricted chimeric gene fragment then was sub-cloned into the EcoRI site of the dephosphorylated pD2 vector to generate the 12.1 Kb plasmid pD2F-G (ATCC 75389).

Example 17:

This Example outlines the preparation of plaque-purified recombinant baculoviruses containing the chimeric $F_{PIV-3}-G_{RSV}$ gene.

Spodoptera frugiperda (Sf9) cells were co-transfected with 2 ug of pD2F-G plasmid DNA (Example 16) and 1 ug of linear wild-type AcNPV DNA (obtained from Invitrogen). Recombinant baculoviruses containing the $F_{PIV-3}-G_{RSV}$ gene were plaque-purified twice according to the procedure outlined in Example 10.

Example 18:

This Example illustrates the presence of the chimeric $F_{PIV-3}-G_{RSV}$ protein in the supernatant of Sf9 and High 5 cells infected with the recombinant baculoviruses.

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Sf9 and High 5 cells were infected with recombinant baculoviruses containing the $F_{PIV-3}-G_{RSV}$ gene (Example 16) at a m.o.i. of 5 to 10 pfu/cell. The supernatant of cells infected with the recombinant viruses tested positive for expressed protein in the PIV-3 F specific ELISA. Supernatants of infected cells reacted with both anti-F PIV-3 and anti-G RSV monoclonal antibodies in immunoblots. These results confirm the presence of the chimeric $F_{PIV-3}-G_{RSV}$ protein in the supernatants of infected Sf9 and High 5 cells.

Example 19:

This Example outlines the preparation of recombinant vaccinia viruses expressing the $F_{PIV-3} - F_{RSV}$ and $F_{RSV} - HN_{PIV-3}$ genes.

Vaccinia virus recombinant viruses expressing the $F_{PIV-3}-F_{RSV}$ (designated vP1192) and $F_{RSV}-HN_{PIV-3}$ (designated vP1195) genes were produced at Virogenetics Corporation (Troy, NY) (an entity related to assignee hereof) using the COPAK host-range selection system. Insertion plasmids used in the COPAK host-range selection system contained the vaccinia K1L host-range gene [Perkus et al., (1990) Virology 179:276-286] and the modified vaccinia H6 promoter [Perkus et al. (1989), J. Virology 63:3829-3836]. In these insertion plasmids, the K1L gene, H6 promoter and polylinker region are situated between Copenhagen strain vaccinia flanking arms replacing the ATI region [open reading frames (ORFs) A25L, A26L; Goebel et al., (1990), Virology 179: 247-266; 517-563]. COPAK insertion plasmids are designed for use in in vivo recombination using the rescue virus NYVAC (vP866) (Tartaglia et al., (1992) Virology 188: 217-232). Selection of recombinant viruses was done on rabbit kidney cells.

Recombinant viruses, vP1192 and vP1195 were generated using insertion plasmids pES229A-6 and PSD.RN, respectively. To prepare plasmid pES229A-6 containing

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the $F_{PIV-3}-F_{RSV}$ gene, the COPAK-H6 insertion plasmid pSD555 was cut with SmaI and dephosphorylated with intestinal alkaline phosphatase. The 2.6 Kb $F_{PIV-3}-F_{RSV}$ gene was retrieved from plasmid pES60A (Example 4) by cutting the
 5 plasmid with EcoRI and BamHI. The 2.6 Kb EcoRI-BamHI $F_{PIV-3}-F_{RSV}$ gene was blunt ended with Klenow polymerase, isolated from a low melting point agarose gel and cloned into the SmaI site of the COPAK-H6 insertion plasmid pSD555 to generate plasmid pES229A-6. This positioned
 10 the $F_{PIV-3}-F_{RSV}$ ORF such that the 5' end is nearest the H6 promoter.

To prepare plasmid PSD.RN, the pSD555 vector first was cut with SmaI and BamHI. Plasmid pM RF-HN (Example 9) containing the truncated $F_{RSV}-HN_{PIV-3}$ gene was cut with
 15 ClaI, blunt ended with Klenow polymerase and then cut with BamHI. The 3.3 Kb $F_{RSV}-HN_{PIV-3}$ gene was cloned into the SmaI-BamHI sites of the pSD555 vector to generate plasmid PSD.RN. This positioned the $F_{RSV}-HN_{PIV-3}$ ORF such that the H6 5' end is nearest the H6 promoter.

20 Plasmids pES229A-6 and PSD.RN were used in in vitro recombination experiments in vero cells with NYVAC (vP866) as the rescuing virus. Recombinant progeny virus was selected on rabbit kidney (RK)-13 cells (ATCC #CCL37). Several plaques were passaged two times
 25 on RK-13 cells. Virus containing the chimeric genes were confirmed by standard in situ plaque hybridization [Piccini et al. (1987), Methods in Enzymology, 153:545-563] using radiolabeled probes specific for the PIV and RSV inserted DNA sequences. Plaque purified virus
 30 containing the $F_{PIV-3}-F_{RSV}$ and $F_{RSV}-HN_{PIV-3}$ chimeric genes were designated vP1192 and vP1195, respectively.

Radioimmunoprecipitation was done to confirm the expression of the chimeric genes in vP1192 and vP1195 infected cells. These assays were performed with lysates
 35 prepared from infected Vero cells [according to the procedure of Taylor et al., (1990) J. Virology 64, 1441-

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1450] using guinea pig monospecific PIV-3 anti-HN and anti-F antiserum and rabbit anti-RSV F antiserum. Both the anti-PIV F and anti-RSV F antisera precipitated a protein with an apparent molecular weight of approximately 90 kDa from vP1192 infected Vero cells. Both anti-RSV F and guinea pig anti-PIV HN antisera precipitated a protein with an apparent molecular weight of approximately 100 kDa from vP1195 infected cells. These results confirmed the production of the $F_{PIV-3}-F_{RSV}$ and $F_{RSV}-HN_{PIV-3}$ chimeric proteins in Vero cells infected with the recombinant poxviruses.

SUMMARY OF DISCLOSURE

In summary of the disclosure, the present invention provides multimeric hybrid genes which produce chimeric proteins capable of eliciting protection against infection by a plurality of pathogens, particularly PIV and RSV. Modifications are possible within the scope of this invention.

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Table 1 Secondary antibody response of guinea pigs immunized with the chimeric $F_{RSV-HN_{PIV-3}}$ protein

Antigen Formulation	Dose (ug)	HAI Titre ^a (log ₂ ± s.e.)		Neutralization Titre ^b (log ₂ ± s.e.)			
		PIV-3		PIV-3		RSV	
		6 wk Bleed	8 wk Bleed	6 wk Bleed	8 wk Bleed	6 wk Bleed	8 wk Bleed
Buffer	-	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0
$F_{RSV-HN_{PIV-3}}$	10.0	9.1 ± 0.3	9.1 ± 0.3	7.1 ± 0.3	7.1 ± 0.5	5.5 ± 0.9	4.5 ± 1.2
	1.0	7.0 ± 2.0	7.3 ± 2.2	5.0 ± 1.5	4.5 ± 1.4	4.5 ± 0.5	3.0 ± 1.0
Live PIV-3		8.6 ± 0.7	7.3 ± 0.6	7.0 ± 0.4	7.3 ± 0.6	N/A	N/A
Live RSV		N/A ^c	N/A	N/A	N/A	5.5 ± 1.5	5.0 ± 1.0

^a Reciprocal mean log₂ serum dilution which inhibits erythrocyte agglutination by 4 hemagglutinating units of PIV-3

^b Reciprocal mean log₂ serum dilution which blocks hemadsorption of 100 TCID₅₀ units of PIV-3 or RSV

^c N/A - not applicable

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TABLE 2

Table 2: Serum antibody response of cotton rats immunized with the chimeric $F_{RSV-HN_{PIV-3}}$ protein^a

Antigen Formulation	Dose (ug)	HAI Titre ^b (log ₂ ± s.d.)		Neutralization Titre ^c (log ₂ ± s.d.)			
		PIV-3		PIV-3		RSV	
		4 wk Bleed	5 wk Bleed	4 wk Bleed	5 wk Bleed	4 wk Bleed	5 wk Bleed
Buffer	-	2.8 ± 0.5	<3.0 ± 0.0	<1.0 ± 1.0	<1.0 ± 0.0	1.8 ± 0.3	0.8 ± 0.7
$F_{RSV-HN_{PIV-3}}$	10.0	9.5 ± 1.3	10.5 ± 0.6	>9.0 ± 0.0	>9.0 ± 0.0	5.2 ± 1.1	5.8 ± 0.9
	1.0	9.3 ± 1.0	10.3 ± 0.5	>9.0 ± 0.0	>9.0 ± 0.0	5.0 ± 0.7	5.8 ± 1.2
Live PIV-3		7.0 ± 0.0	8.5 ± 0.7	>9.0 ± 0.0	9.2 ± 0.7	N/A	N/A
Live RSV		N/A ^d	N/A	N/A	N/A	5.5 ± 0.6	8.5 ± 0.6

^a Each value represents the mean titre of antisera from 8 animals.

^b Reciprocal mean log₂ serum dilution which inhibits erythrocyte agglutination by 4 hemagglutinating units of PIV-3

^c Reciprocal mean log₂ serum dilution which blocks hemadsorption of 100 TCID₅₀ units of PIV-3 or RSV

^d N/A - not applicable

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Table 3. Response of immunized cotton rats to PIV/RSV challenge^a

Antigen Formulation	Dose (ug)	Mean virus lung titre log ₁₀ /g lung ± s.d.	
		RSV	PIV-3
Buffer	-	3.7 ± 0.3	3.4 ± 0.3
F _{RSV} -HN _{PIV-3}	10.0	≤1.5 ± 0.0	≤1.5 ± 0.0
F _{RSV} -HN _{PIV-3}	1.0	≤1.5 ± 0.0	≤1.5 ± 0.0
Live RSV		≤1.5 ± 0.0	≤1.5 ± 0.0
Live PIV-3		≤1.5 ± 0.0	≤1.5 ± 0.0

^a Animals were challenged intranasally with 100 TCID₅₀ units of PIV-3 or RSV and killed 4 days later. Each value represents the mean virus lung titre of 4 animals.

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